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Cloning and expression of a cDNA for the human prostacyclin receptor

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Abstract

A functional cDNA for the human prostacyclin receptor was isolated from a cDNA library of CMK cells, a human megakaryocytic leukaemia cell line. The cDNA encodes a protein consisting of 386 amino acid residues with seven putative transmembrane domains and a deduced molecular weight of 40,956. [^{3}H]Iloprost specifically bound to the membrane of CHO cells stably expressing the cDNA with a K_d of 3.3 nM. This binding was displaced by unlabelled prostanoids in the order of iloprost = cicaprost >> carbacyclin > prostaglandin E_1 (PGE_1) > STA_2. PGE_2, PGD_2 and $PGF_{2\alpha}$ did not inhibit it. Iloprost in a concentration-dependent manner increased the cAMP level and generated inositol trisphosphate in these cells, indicating that this human receptor can couple to multiple signal transduction pathways.

Key words: Prostaglandin; Prostacyclin; Prostanoid receptor; Signal transduction

1. Introduction

Prostacyclin (PGI_2) is an unstable metabolite of arachidonic acid produced by endothelial cells [1]. It is a potent inhibitor of platelet aggregation, and bronchial and vascular smooth muscle contraction. These actions can counteract the proaggregatory and vasoconstrictor activities of another prostanoid, thomboxane (TX) A_2, and contribute to the maintenance of homeostasis in circulation [2]. These activities of PGI_2 have been regarded as being useful for preventing and managing diseases such as cerebral thrombosis and myocardial infarction. For the development of PGI_2 analogues as therapeutic agents for cardiovascular and circulatory disease, it is necessary to elucidate the precise mechanism of PGI_2 actions in these tissues. The actions of PGI_2 were presumed to be mediated by a cell surface receptor [3,4]. The PGI_2 receptor has been detected on human platelets through radioligand binding assays involving [^{3}H]iloprost, a stable analogue of PGI_2 [5–7], and on MEG-01 cells, a human megakaryocytic leukaemia cell line [8]. Furthermore, several PGI_2 analogues have been reported to have PGI_2-like actions on platelets from human but not from rat or rabbit, suggesting the existence of species difference in PGI_2 receptor [4,9,10]. However, the PGI_2 receptor has not been isolated, and its molecular characterization has been carried out only poorly. Recently, we cloned and characterized the mouse PGI_2 receptor [11]. In order to elucidate the structural and functional characteristics of human receptor, we performed cDNA cloning and expression analysis of the human PGI_2 receptor.

2. Materials and methods

2.1. Molecular cloning of a cDNA for the human PGI_2 receptor

A human cDNA library of CMK cells, a human megakaryocytic leukaemia cell line [12], was constructed by means of an oligo(dT) priming method, and screened by hybridization with a partial fragment of the mouse PGI_2 receptor cDNA [11]. Hybridization was carried out at 56°C for 15 h in 6 × SSC (900 mM NaCl and 90 mM sodium citrate) containing 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin) and 0.5% sodium dodecyl sulfate, and filters were washed twice at 56°C for 30 min in 2 × SSC containing 1% sodium dodecyl sulfate. One positive clone, MK71, was isolated, and subjected to sequence and expression analysis. Nucleotide sequencing was carried out on both strands by the dideoxy chain termination method.

2.2. Expression in CHO cells and ligand binding assay

Establishment of chinese hamster ovary (CHO) cells stably expressing the receptor was performed essentially as described previously [13]. the *Eco*RI insert of MK71 was subcloned into pdKCR-dhfr [14], and the resultant plasmid DNA was transfected to CHO cells deficient in dihydrofolate reductase activity (CHO-dhfr$^-$) by the lipofection method [15]. Cell populations expressing the cDNA together with dihydrofolate reductase were selected in the α-modification of Eagle's medium lacking ribonucleosides and deoxyribonucleosides and containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% dialyzed fetal bovine serum (Cell Culture Laboratories). From these cell populations, clonal cell lines were isolated by single-cell cloning. Expression of the human cDNA was assessed by Northern blot analysis. The crude membrane fraction (100,000 × *g* pellet fraction) of the clonal cells was used for the

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Sequence data in this article have been deposited with the GenBank/EMBLE/DDBJ Data Libraries under accession no. D25418.

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[^{3}H]iloprost (Amersham) binding assay. [^{3}H]Iloprost binding was examined by incubating 50μg protein of the crude membrane in the suspension buffer (100 μl) with 20 nM (in displacement experiments) or various concentrations (in Scatchard analysis) of [^{3}H]iloprost at 30°C for 1 h as described previously [11].

2.3. cAMP assay and measurement of [^{3}H]IP$_3$ formation

A CHO cell clone showing both mRNA expression and [^{3}H]iloprost binding was seeded at 5×10^4/well in a 24-well plate and cultured for 72 h. Cells (5×10^5 cells/well) were washed with 0.5 ml of *Hepes*-buffered saline (HBS; 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, and 15 mM HEPES-NaOH, pH 7.4) and preincubated for 10 min in 450 μl of the solution containing 1 mM 3-isobutyl-1-methylxanthine at 37°C. Iloprost in 50 μl of the same solution was then added and incubated for 10 min at 37°C. The reaction was terminated by addition of 500 μl of 10% trichloroacetic acid and the cAMP level was determined by radioimmunoassay using a cAMP[^{125}I] assay system (Amersham).

For measurement of [^{3}H]inositol trisphosphate (IP$_3$) formation, the CHO cell clone was seeded at 3.0×10^5/well in 6-well plates, cultured for 48 h, and then labelled with 1 μCi/ml myo-[2-^{3}H]inositol for 12 h [16]. Cells were washed twice with HBS and preincubated in 2 ml of HBS containing 10 mM LiCl for 5 min at 37°C. The solution was aspirated and the reaction was started by adding 1 ml of HBS containing 10 mM LiCl and various concentrations of iloprost. After incubation at 37°C for 2 min, the medium was quickly aspirated and 1 ml of 5% trichloroacetic acid was added. Measurement of [^{3}H]IP$_3$ formed was performed as described previously [11].

3. Results and discussion

Fig. 1 shows the nucleotide and deduced amino acid sequences of MK71. MK71 contains a 2.0 kb insert which has an open reading frame of 1,158 base pairs. The deduced amino acid sequence shows that it encodes a protein of 386 amino acids with an estimated molecular weight of 40,956. The hydrophobicity profile obtained by Kyte and Doolittle's method [17] indicated that it possesses seven hydrophobic segments, suggesting that it is a guanine nucleotide-binding protein-coupled rhodopsin-type receptor. Two potential N-glycosylation sites [18] were found in the N-terminal and the first extracellular loop regions.

Membranes of CHO cells expressing the receptor showed specific binding of [^{3}H]iloprost, a specific radioligand for the PGI$_2$ receptor. Scatchard analysis of this binding yielded a dissociation constant of 3.3 nM and maximal binding of 3.2 pmol/mg protein. Fig. 2 shows the specificity of this binding. Specific [^{3}H]iloprost binding was displaced by unlabelled prostanoids in the order of iloprost = cicaprost >> carbacyclin, all of which are PGI$_2$ agonists, > prostaglandin E$_1$ (PGE$_1$) > STA$_2$, a TXA$_2$ agonist. PGE$_2$, PGD$_2$ and PGF$_{2\alpha}$ did not inhibit it. This order agrees well with that observed in a displacement study on human platelets [7,9]. These results demonstrate that MK71 encodes the human PGI$_2$ receptor.

On signal transduction analysis of the mouse PGI$_2$ receptor, we found that the receptor can couple to multiple signalling pathways, adenylate cyclase and phospholipase C. We next examined the existence of this multiple signalling in the case of the human receptor. As shown in Fig. 3a, iloprost generated cAMP in a concentration-dependent manner in CHO cells stably expressing the receptor. Furthermore, as shown in Fig. 3b, iloprost gen-

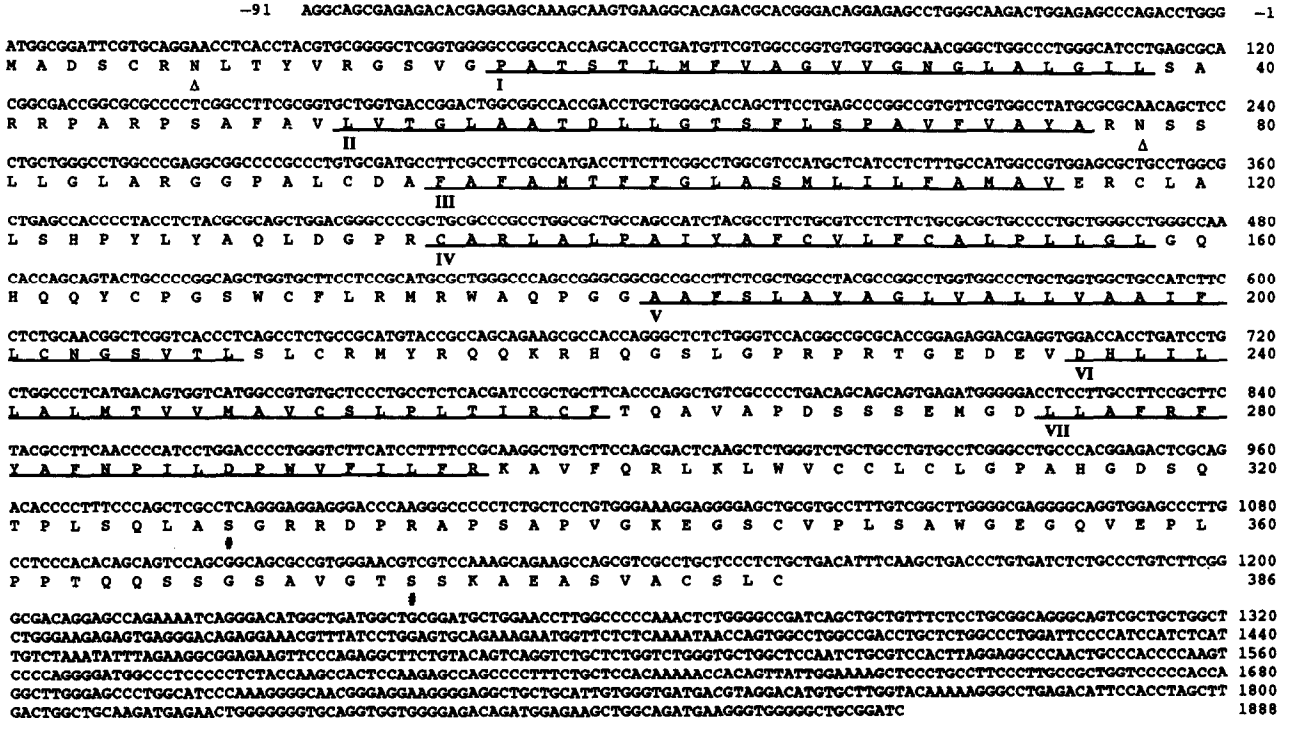

Fig. 1. Nucleotide and deduced amino acid sequences of MK71. The deduced amino acid sequence is shown beneath the nucleotide sequence using a single-letter code. The positions of the putative transmembrane segments, I–VII, are underlined. Triangles, potential N-glycosylation sites; parallel crosses, potential sites of phosphorylation by protein kinase C.

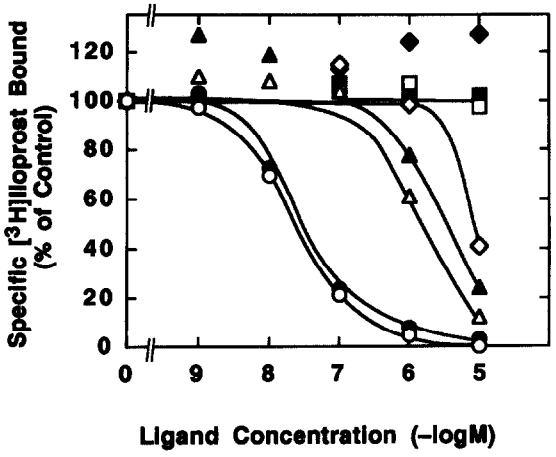

Fig. 2. Displacement of the specific binding of [^{3}H]iloprost in membranes of CHO cells expressing MK71. Membranes of CHO cells expressing MK71 were incubated with 20 nM [^{3}H]iloprost and various unlabelled prostanoids at the indicated concentrations. Unlabelled prostanoids are indicated as follows. ○, iloprost; ●, cicaprost; △, carbacyclin; ▲, PGE_1; □, PGE_2; ■, PGD_2; ◇, STA_2; ◆, $PGF_{2\alpha}$.

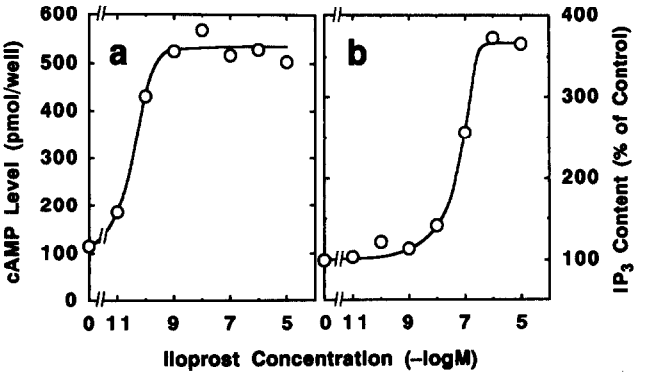

Fig. 3. Effect of iloprost on the cAMP level (a) and IP_3 formation (b) in MK71-expressing CHO cells. CHO cells were incubated with the indicated concentrations of iloprost, and then the cAMP level and [^{3}H]IP_3 formation were determined as described in section 2. The results shown are the means of triplicate determinations.

erated [^{3}H]IP_3 in a concentration-dependent manner in CHO cells expressing the receptor, suggesting that it evoked phosphatidylinositol hydrolysis. In previous studies, PGI_2 or its analogues evoked an increase in the cytosolic Ca^{2+} concentration in human cell lines, MEG-01 cells [8], and HEL cells, a pluripotent human erythroleukaemia cell line [19]. The present study revealed that the human PGI_2 receptor as well as the mouse receptor can couple to multiple signalling pathways, adenylate cyclase and phospholipase C.

Fig. 4 shows a comparison of the amino acid sequences of the human and mouse PGI_2 receptors. The sequence of the human PGI_2 receptor is 73% identical to that of the mouse receptor. This sequence identity between the spieces is relatively lower than that observed in other G protein-coupled receptors. Furthermore, one of the structural features of the human PGI_2 receptor is deletion of the N-terminal first 30 amino acids observed in the mouse receptor. Although we could not detect the difference in binding characters of the human and mouse receptors in this study, it is likely that two PGI_2 receptors may show different specificities of binding in the case of using other kinds of PGI_2 analogues. In fact, several PGI_2 analogues have been reported to have PGI_2-like actions on platelets from human but not from rat or rabbit, suggesting the existence of species difference in PGI_2 receptor [4,9,10]. Further investigations should be

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                                                                I
human IP   ------------------------------MADSCRNLTYVRGSVGPATSTLMFVAGVVG   30
mouse IP   MKMMASDGHPGPPSVTPGSPLSAGGREWQG**G**W*I***QD*****************   60

           II                                                  III
NGLALGILSARRPARPSAFAVLVTGLAATDLLGTSFLSPAVFVAYARNSSLLGLARGGPALCDAFAFAMT  100
********G***RSH***********V******C*******************H**TM***T******  130

                    IV
FFGLASMLILFAMAVERCLALSHPYLYAQLDGPRCARLALPAIYAFCVLFCALPLLGLGQHQQYCPGSWC  170
******T*****************************F***S*****C***S*******E**********  200

           V                                                   VI
FLRMRWAQPGGAAFSLAYAGLVALLVAAIFLCNGSVTLSLCRMYRQQKRHQGSLGPRPRTGEDEVDHLIL  240
*I***S*****C********S*M*****TS**F*********YH*****R**H**FV*TS*AR****Y****  270

                    VII
LALMTVVMAVCSLPLTIRCFTQAVAPDSSSEMGDLLAFRFYAFNPILDPWVFILFRKAVFQRLKLWVCCL  310
******I********M**G****I****-*R**********N**********************F*L***  339

CLGPAHGDSQTPLSQLASGRRDPRAPSAPVGKEGSCVPLSAWGEGQVEPL--PPTQQSSGSAVGTSSKAE  378
*ARSV***L*A***RP*******P**TSLQA****W****S**T***A**TAV*LTGGD*CS**MP**S*  409

ASVACSLC  386
*IA*****  417
```

Fig. 4. Comparison of the amino acid sequences of the human (upper) and mouse (lower) PGI_2 receptors. Identical amino acid residues are indicated by asterisks and non-identical residues are shown in the mouse sequence. The seven transmembrane domains are indicated above the amino acid sequences.

Fig. 5. Comparison of the amino acid sequences among human prostanoid receptors. The deduced amino acid sequences of the human PGI_2 receptor (hIP), human EP_2 (hEP_2), human EP_3 (hEP_3), human EP_1 (hEP_1), human PGF receptor (hFP), and human TXA_2 receptor (hTP) are shown aligned to optimize homology. Identical amino acid residues in four or more sequences are indicated by bold characters. The seven transmembrane domains are indicated above the amino acid sequences.

made to conclude whether the spieces difference exists or not in the PGI_2 receptor.

Fig. 5 shows a comparison of the amino acid sequences among the cloned human prostanoid receptors. The human IP receptor shares 37% overall amino acid identity with human EP_2 [20] receptor and 34, 31, 29, and 28% identity with human TP [21], EP_1 [22], EP_3 [23], and FP [24] receptors, respectively. The amino acid identity is mainly restricted to the transmembrane domains, especially the VII, which is the most conserved, with the V being the least conserved. The conserved residues are presumed to be important in the recognition of the common sturctures in prostanoid ligands or in the formation of the receptor structure. For instance, Arg-279, conserved among all eicosanoid receptors, has been proposed to be the counter-ligand for the prostanoid carboxyl moiety [13,21]. Among these prostanoid receptors, the human IP receptor has the common sequences especially with EP_2 receptor; GVVGN in the I, VLFCALP in the IV, and YAG in the V. Furthermore, within the most highly conserved region in the VII, proline 285 is conserved only in the IP and EP_2 receptors, while the other prostanoid receptors have glutamine residue for proline. Therefore, the human IP receptor is most highly related to the EP_2 receptor, and it is interesting that these two receptors can couple to the stimulation of adenylate cyclase [20,25]. The human IP receptor also shares several of the features common to all other G protein-coupled receptors which are thought to be important in defining the topography and functions of this family [26]. For instance, there is one potential N-glycosylation site (Asn-7) in the putative extracellular amino terminus (Fig. 1), and aspartic acid (Asp-60) in the domain II. Conserved cysteine residues are found in the extracellular loops 1 and 2 (Cys-92 and Cys-170). The intracellular loops and the carboxyl terminal tail contain a number of serine and threonine residues (Fig. 1), potential phosphorylation sites, which may be involved in receptor desensitization [27].

In summary, we presented here the structure and function of the human PGI_2 receptor. The results of this study will be very useful for designing specific PGI_2 agonists or antagonists for therapeutic purposes in humans.

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